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The RecB helicase-nuclease tether mediates Chi hotspot control of RecBCD enzyme

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ABSTRACT

In bacteria, repair of DNA double-strand breaks uses a highly conserved helicase–nuclease complex to unwind DNA from a broken end and cut it at specific DNA sequences called Chi. In *Escherichia coli* the RecBCD enzyme also loads the DNA strand-exchange protein RecA onto the newly formed end, resulting in a recombination hotspot at Chi. Chi hotspots regulate multiple RecBCD activities by altering RecBCD's conformation, which is proposed to include the swinging of the RecB nuclease domain on the 19-amino-acid tether connecting the helicase and nuclease domains. Here, we altered the tether and tested multiple RecBCD activities, genetically in cells and enzymatically in cell-free extracts. Randomizing the amino-acid sequence or lengthening it had little effect. However, shortening it by as little as two residues or making substitutions of ≥10 proline or ≥9 glycine residues dramatically lowered Chi-dependent activities. These results indicate that proper control of RecBCD by Chi requires that the tether be long enough and appropriately flexible. We discuss a model in which the swing-time of the nuclease domain determines the position of Chi-dependent and Chi-independent cuts and Chi hotspot activity.

INTRODUCTION

When DNA in a cell is broken, it must be repaired or the cell dies. Consequently, all living organisms have evolved mechanisms to repair DNA damage, often through the combined activity of helicases and nucleases. Repair of DNA double-strand (ds) breaks involves processing the ds end to produce a long single-strand (ss) end coated with a protein that promotes exchange of this strand with its homolog in intact DNA (reviewed in reference (1)). In bacteria, the three-subunit enzyme RecBCD or its analog the two-subunit enzyme AddAB binds to ds DNA ends and rapidly unwinds the DNA (2–4) (Figure 1A). Upon encountering a properly oriented special sequence called Chi, these enzymes (e.g., *Escherichia coli* RecBCD and *Bacillus subtilis* AddAB) cut the DNA near this sequence (5–7). RecBCD actively loads the DNA strand-exchange protein RecA onto the newly formed ss end at Chi (8). The RecA-ssDNA filament exchanges position with the corresponding strand in intact ds DNA to form a joint DNA molecule (8). Joint molecules include the initial displacement (D)-loop formed by strand exchange and a subsequent structure (Holliday junction) formed by an additional strand cutting and strand annealing (1). Two intact ds DNA molecules can be formed either by replication initiated at the D-loop or by resolution of the Holliday junction by the RuvC class of enzymes (1). If the broken and intact ds DNA molecules differ genetically, recombinant DNA molecules can be formed. Here, we elucidate how Chi hotspots control RecBCD enzyme to repair broken DNA and thereby to maintain cell viability and generate genetic diversity.

RecBCD enzyme of *E. coli* is a complex three-subunit enzyme with multiple activities broadly grouped as helicase and nuclease. RecBCD has two helicases—RecB, which travels in the 3′ → 5′ direction on one strand, and RecD, which travels 5′ → 3′ on the other strand (9). The RecB and RecD subunits each contain an adenosine triphosphatase (ATPase) site within their helicase domains (10,11); these subunits are held together by the RecC subunit (12) (Figure 1B). RecB also has a nuclease domain, whose activity in the holoenzyme depends on the other subunits and is regulated by Chi hotspots (5,13–14). During unwinding of DNA, RecBCD generates a 3′-ended ssDNA tail when the enzyme encounters a Chi hotspot sequence from the proper direction (6). In *E. coli*, Chi requires the core sequence 5′ GCTGGTGG 3′, which is equated with Chi, but the activity of this site depends strongly on nucleotides 4–7 to its 3′ side (15–17).

In reactions with purified components, the activities of RecBCD enzyme are changed at Chi in a manner that depends on the reaction conditions. When the concentration of ATP is greater than that of Mg^{2+} ions, RecBCD unwinds the DNA and nicks the Chi-containing strand of DNA a few nucleotides to the 3′ side of the core sequence (5,6); continued unwinding produces 3′-ended ss DNA for strand ex-

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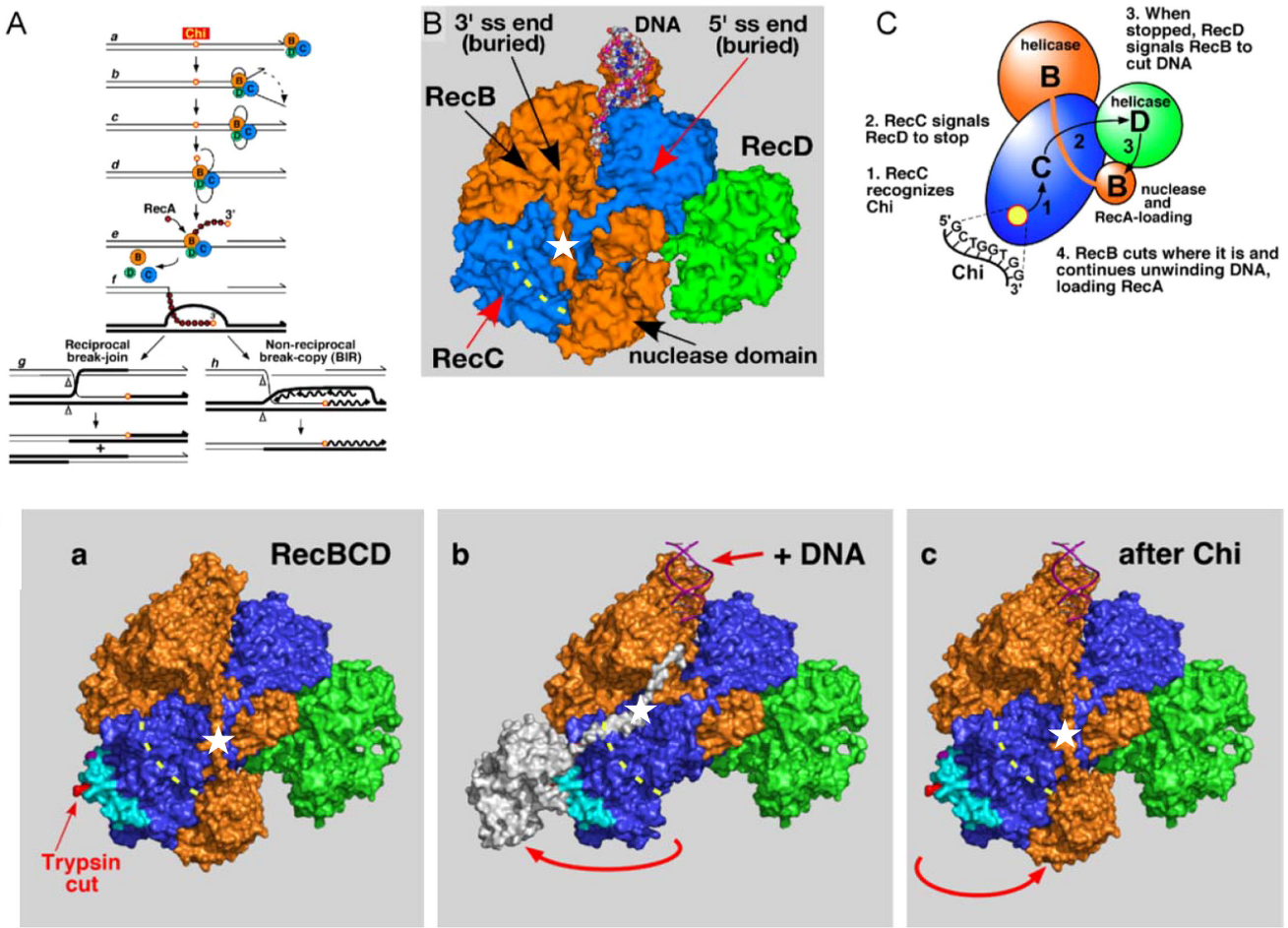

Figure 1. Models for RecBCD enzyme and its Chi-dependent promotion of DNA break repair and genetic recombination. (**A**) RecBCD pathway of genetic recombination (from (30)). RecBCD binds a ds DNA end (*a*) and unwinds the DNA with the production of enlarging ss DNA loops, because the RecD helicase moves faster than the RecB helicase (9) (*b*-*c*). If the 3′ and 5′ tails anneal, two loops are formed, but the tails may be kept apart by single-strand binding (SSB) protein (2,64). Upon encountering Chi, RecBCD cuts the strand with 5′ GCTGGTGG 3′ (5,6) (*d*), loads RecA onto the newly formed 3′ ss DNA end (8) (*e*) and later disassembles into the three inactive subunits (31) (*f*). The RecA-ssDNA filament invades intact ds DNA to form a D-loop (8) (*f*), which can be converted into a Holliday junction (*g*), which is resolved into reciprocal recombinants, or prime DNA replication (*h*), which produces a non-reciprocal recombinant and a parental-type DNA (1) (not shown). (**B**) Crystal structure of RecBCD bound to a ds DNA hairpin (12) (adapted from (30)). RecB (orange) contains helicase and nuclease domains connected by a 19-amino-acid tether (white star). RecD (green) is held to RecB via RecC (blue) and possibly by unstructured RecD amino acids not shown (35). The 3′-ended strand likely passes through a tunnel (yellow dashed line) in RecC and into the nuclease active site when Chi is encountered. (**C**) Signal-transduction model for Chi's control of RecBCD enzyme (from (30)). When Chi is in the RecC tunnel, RecC signals RecD to stop unwinding DNA. RecD then signals RecB to nick the DNA and to begin loading RecA. (**D**) Nuclease-swing model for Chi's control of RecBCD enzyme (adapted from (33)). Before DNA is bound, RecBCD in solution assumes its conformation in the published structures (12,35) (a). Upon binding DNA, the nuclease domain swings away from the exit of the RecC tunnel (33) (b). When Chi is encountered during unwinding, the nuclease domain swings back, cuts the DNA at Chi and begins loading RecA protein, perhaps after rotating to prevent further nuclease action (c).

change by RecA (6). When the concentration of Mg^{2+} ions is greater than that of ATP, RecBCD makes, during unwinding, occasional endonucleolytic nicks up to Chi on the 3′-ended strand, releasing fragments hundreds of nucleotides long; continued unwinding and similar nicking of the complementary strand produces 3′-ended ss DNA for strand exchange (18–20). A second round of RecBCD action is required to produce the acid-soluble, short oligonucleotides typically assayed as 'nuclease activity' with purified enzyme (21–24) and in repair-deficient cells (e.g., *recA* mutants) after DNA damage (25). Note that RecBCD is called an 'exonuclease' because it requires a DNA end (ds or ss) for activity, but it makes only endonucleolytic scissions on DNA. Analysis of abundant genetic and physiological data indicates that in living *E. coli* cells the initial RecBCD enzyme simply nicks the DNA at Chi rather than repeatedly nicking the DNA up to Chi and switching the strand nicked (see (1) for review of data). Data presented here suggest a mechanistic reconciliation of the two reactions by the initial RecBCD enzyme molecule (see 'Discussion' section).

Mutations altering amino acids lining a tunnel in RecC (Figure 1B) alter Chi activity but leave other activities more or less intact (15,26–29). This feature and the properties of special *recB* mutations led to the signal transduction model (Figure 1C), which posits that when the Chi octamer is in the RecC tunnel it signals, via RecC, then RecD and then

RecB, the nuclease to cut the Chi-containing strand (30). Cutting occurs 4–6 nt to the 3′ side of the core octamer (6). The dependence of Chi activity on the 3′ flanking nucleotides may reflect a preference for the nuclease to cut within certain nucleotide sequences (15,16). Cutting of this strand ceases after one Chi is cut (31,32); it has been hypothesized that the nuclease rotates to enable loading of RecA while effectively inactivating the nuclease (33). Additional DNA reactions lead to repaired, and potentially recombinant, DNA (Figure 1A).

A central question about RecBCD's repair of DNA is how the enzyme is controlled by Chi. RecBCD undergoes profound conformational changes during its reaction cycle (33). In solution, in the absence of DNA the enzyme appears to have the conformation of that in crystal and cryoEM structures of the enzyme bound to DNA—a patch of amino acids on the surface of RecC is sensitive to proteases, and the small angle X-ray scattering (SAXS) profile fits the published structures with the DNA computationally removed (12,33–35) (Figure 1D). Upon addition of DNA, the enzyme in solution becomes resistant to proteases at the RecC patch and the SAXS profile shows mass moving from one area to another. These results are consistent with the nuclease domain moving to allow the RecC patch to be protease-sensitive without DNA but resistant after its addition. After encountering Chi, the RecC patch becomes protease-sensitive again, suggesting that the nuclease domain has returned to its position in the published structures, where it can cut DNA to the 3′ side of Chi.

The observations above are accounted for by the nuclease-swing model (33), in which the RecB nuclease domain undergoes a large conformational change mediated by a 19-amino-acid tether connecting its nuclease and helicase domains (12). The tether, seen in crystal and cryoEM structures (Figure 1D), is hypothesized to direct the nuclease domain from one side of RecBCD to another, depending on the presence of DNA and the encounter with Chi as described above (12,33–35). In the published structures this tether is fully stretched out and appears to be just long enough to allow the nuclease domain to be positioned at the exit of the RecC tunnel, where the nucleotides flanking Chi would be when they are cut (6,12) (see Figure 2). Thus, the tether length may be important for Chi's control of RecBCD. During the RecBCD reaction cycle (Figure 1D) the nuclease domain is hypothesized to swing (33), and the flexibility of the tether may also be important to direct the nuclease domain from one position to another. Here, we test these predictions by an extensive mutational analysis of the tether. Our results support the nuclease-swing model, which may be related to large conformational changes of other enzymes.

MATERIALS AND METHODS

Bacterial strains, phage and plasmids

Bacterial strains, phage and plasmids are listed in Supplementary Table S1 with their genotypes and sources. Plasmids are derivatives of plasmid pSA607, which encodes fully active RecBCD enzyme from *recB*$^+$, *recD*$^+$, and *recC2773* (*recC* with six histidine codons at the C terminus) (33). RecBCD phenotypes were determined in transformants of *E. coli* strain V2831 which carries a chromosomal *recBCD* deletion (*ΔrecBCD2731*).

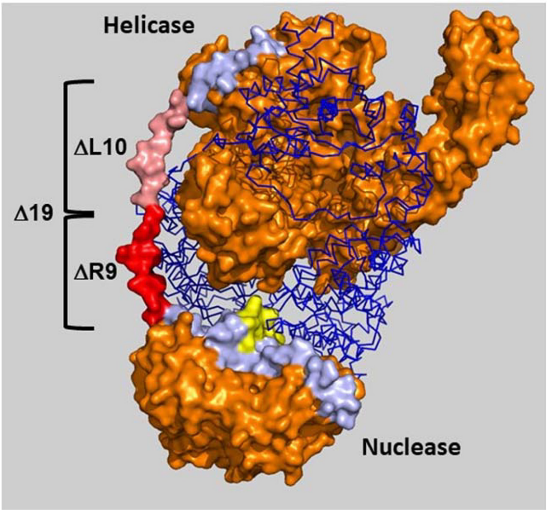

Figure 2. RecB (orange) with its tether connecting the helicase and nuclease domains. The 'tether' (amino acids 881–899 with 881–890 in salmon and 891–899 in red), defined here, is included in the 'linker' region (amino acids 870–940; gray), defined in refs. (12,35). The extents of three deletions studied here are shown. The linker also contains an α-helix (amino acids 913–922; yellow), defined in ref. (12). The RecC subunit (blue in ribbon representation) is in direct contact with much of RecB, including the tether.

Growth media

Tryptone broth, bottom and top agar, tryptone agar plus yeast extract, LB broth and agar, and suspension medium have been described (15,29,36). Cell-free extracts were prepared after growth in Terrific Broth (Thermo Fisher Scientific, Waltham, MA). Plasmid-containing strains were grown in the presence of ampicillin (100 μg/ml).

Construction of mutants with altered RecB tethers

Mutations altering the tether of RecB (Supplementary Table S2) were produced on plasmid pSA607 using the QuikChange Site-Directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) or the Q5 Site Directed mutagenesis kit (New England Biolabs, Ipswich, MA, USA) with oligonucleotides (Integrated DNA Technologies, Coralville, IA, USA). Oligonucleotides with the desired mutations were designed using the web-based QuikChange Primer Design Program (https://www.genomics.agilent.com/primerDesignProgram.jsp) or NEBaseChanger (https://nebasechanger.neb.com); sequences are available on request. The presence of the desired mutation was confirmed by sequencing at least 300 base pairs on each side of the targeted change.

Phage test screen of nuclease activity

RecBCD nuclease blocks the growth and plaque formation of phage T4 gene *2* mutants, which lack a protein that binds to the end of infecting DNA, protecting it from degradation (37). Consequently, wt T4 (*2*$^+$) but not T4 *gene 2* mutant

(2^-) phage form plaques on strains with RecBCD nuclease activity; both phages make plaques on strains lacking RecBCD nuclease activity (26,29). Plasmid transformants of strain V2831 (*ΔrecBCD2731*) were grown overnight at 37° in tryptone broth. Bacteria (100 μl of saturated culture) were added to 2.5 ml of top agar and the mixture poured evenly over the surface of a bottom agar plate. After the top agar hardened, 10 μl aliquots of phage T4 2^+ or T4 2^- were spotted on the surface of the plate. These spots produced between 50 and 150 plaques on strain V2831 with plasmid pSA21 (*recB21;* nuclease-negative, Nuc$^-$) (Supplementary Figure S1).

Genetic recombination assays

Lambda vegetative crosses and determination of recombinant frequency and Chi hotspot activity were as described (38) using λ 1081 × 1082 (cross 1) and λ 1083 × 1084 (cross 2). J^+ R^+ recombinants were selected on strain 594 (Su$^-$) at 39°C and scored as clear or turbid; total phage were assayed on strain C600 (Su$^+$). J^+ R^+ recombinant frequency is the titer on 594 divided by that on C600, using the mean of the two crosses. For Chi hotspot activity, plaques on 594 were examined for recombination in an interval with Chi and in another without Chi, readily seen as turbid (*cI*$^+$) and clear (*cI857*) plaques among the selected J^+ R^+ phage. The turbid:clear ratio is assayed with Chi in one interval (*cI – R*; cross 1) or in the other (*J – cI*; cross 2) and the root-mean-square of this ratio (for cross 1) and its reciprocal (for cross 2) is taken as Chi hotspot activity; note that the Chi-free interval normalizes the results, so that a hotspot value of 1 means Chi has no effect on the distribution of recombination events. Thus, Chi hotspot activity $= \sqrt{(t/c_1) \div (t/c_2)}$, where t/c_1 is the ratio of turbid (c^+) to clear (*cI857*) plaques from cross 1, and t/c_2 from cross 2, among J^+ R^+ recombinants.

Escherichia coli Hfr crosses were conducted as described (29). The Hfr donor was strain S727 (Hfr PO44); recipients were plasmid-containing derivatives of strain V2831 (*hisG4 rpsL31 ΔrecBCD2731*). His$^+$ [Str^R] recombinants were selected on minimal medium lacking histidine and containing streptomycin. Frequencies are expressed as the titer of His$^+$ [Str^R] exconjugants divided by the Hfr titer in the cross, which used a ratio of 1 Hfr per ∼10 F$^-$ recipient cells. Data are expressed as a fraction of the recombinant frequency (5.1 ± 0.31%) for the *recBCD*$^+$ (wt) strain in each cross.

Preparation of cell-free extracts and RecBCD enzymatic assays

Plasmid transformants of strain V2831 were grown in Terrific Broth to late log phase. Extracts were prepared as described (39) and assayed for ATP-dependent solubilization of uniformly ^{3}H-labeled T7 DNA as described (40). Each 20-min assay used at least two protein concentrations that gave a linear relationship between solubilized DNA and protein.

The substrate for DNA unwinding and Chi-dependent nicking was HindIII-linearized pBR322 χ^o or χ^+*F225*. Linearized DNA was treated with shrimp alkaline phosphatase (New England Biolabs) and labeled at the 5′ end with γ-^{32}P ATP (3000 Ci/mmol; Perkin Elmer, Waltham, MA, USA) using T4 polynucleotide kinase (New England Biolabs). Unincorporated nucleotides were removed from the substrate by an SR200 minicolumn (GE Healthcare, Chicago, IL, USA).

DNA unwinding and Chi-dependent nicking were assayed in 15 μl reactions containing 25 mM Tris-acetate (pH 7.5), 5 mM ATP, 2.5 mM magnesium chloride, 1 mM DTT, 1 μM SSB (Promega, Madison, WI, USA), and 3 nM DNA. Reactions were for 2 min at 37° with the amount of extract protein indicated on the figures. Reactions were stopped by the addition of 5 μl of S buffer (0.1 M ethylenediaminetetraacetic acid, 2.5% sodium dodecyl sulphate (SDS), 10% sucrose, 0.125% bromphenol blue, and 0.125% xylene cyanol). Products of the reaction were separated on 0.9% agarose gels (22 cm long) in Tris-Acetate Electrophoresis Buffer (41), run at 120 volts for 2 h and visualized on a Typhoon Trio Phosphorimager (GE Healthcare Lifesciences).

Detection of RecBCD enzyme in cell-free extracts

RecBCD enzymes with wild-type (wt) or mutant RecB tethers were analyzed in extracts by western blot. Extract proteins were separated on denaturing or native NuPage Novex 3–8% polyacrylamide Tris-acetate gels (Invitrogen, Carlsbad, CA, USA) (7 cm long). Native gels were run at 120 volts for 2 h in 100 mM Tris-acetate buffer (pH 7.5). Denaturing gels were run at 150 volts for 1 h in Tris-acetate SDS running buffer (50 mM Tricine, 50 mM Tris base, 0.1% SDS; pH 8.2). Proteins were transferred to PVDF membranes (Immobilon-FL, Millipore Sigma, St Louis, MO, USA) and probed with polyclonal rabbit antibodies specific for RecC or RecB (31). Antibodies were detected by Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen) and imaged on an Odyssey LI-Cor IR scanner (LI-COR, Lincoln, NE, USA).

RESULTS

A region of RecB encompassing the tether is essential for RecBCD activities

A 'linker' region of ∼70 amino acids connecting the RecB helicase and nuclease domains was identified in a crystal structure of RecBCD bound to DNA (Figures 1B and 2) (12,35). This region, between amino acids 870–940, contains an α-helix (amino acids 913–922) proposed to be essential in regulating nuclease activity (see below) (12,35) and a protease-sensitive cleavage site (amino acids 928–933) used to identify and purify the nuclease domain (13,42). This linker region includes 19 amino acids (881–899) that we define as the 'tether,' a nearly fully extended amino-acid chain between the globular structures of the helicase and nuclease domains (Figure 2).

To test the importance of the tether and its surrounding amino acids, we made a series of deletions and tested various activities of RecBCD in cells. We measured recombination proficiency and hotspot activity in a standard 'hotspot' cross of two lambda phages (see 'Materials and Methods' section for details) (38). In wt (*recBCD*$^+$) cells Chi activity was 5.1 ± 0.1, indicating that Chi increases the recombinant frequency in the interval in which it resides by

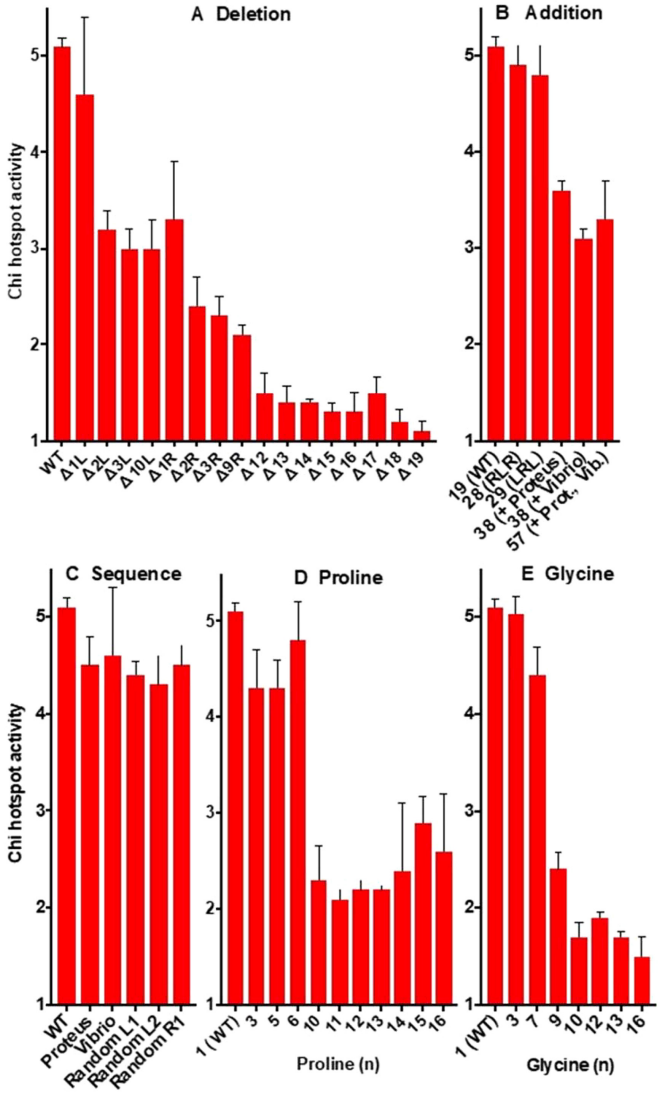

Figure 3. Chi hotspot activity in RecB tether mutants. Strains are transformants of strain V2831 (*ΔrecBCD2731*) with the indicated *recB* tether mutation on a plasmid (see Supplementary Table S2 for complete descriptions). Chi hotspot activity was determined as described (38) and is 5.1 ± 0.1 for *recBCD*$^{+}$ (WT) and 0.99 ± 0.02 for *recB21* (null mutant). Data are the mean of two experiments with the indicated range (|) or the mean ± SEM (⊤) from 3 to 17 independent experiments. Mutants are grouped by type: (**A**) deletion of amino acids; (**B**) addition of amino acids; (**C**) amino acid sequence changes; (**D**) substitutions with proline; (**E**) subsititutions with glycine.

a factor of about five (Table 1 and Figure 3A). The RecBCD null mutant was recombination-deficient and Chi was essentially completely inactive; Chi hotspot activity was not significantly different from one ($P = 0.57$ by one sample *t*-test) since the frequency of recombinants in an interval was the same with and without Chi (Table 1). We deleted amino acids 877–929 of RecB (i.e., most of the linker region noted above); this mutant was recombination-deficient and lacked Chi hotspot activity (Tables 1 and 2), demonstrating that this region, or a portion of it, is essential for producing recombinants. Next, we deleted amino acids 913–922, which encompass the α-helix noted above (12,35) and found that the mutant was recombination proficient and had high Chi activity, similar to that in wt (Table 1 and Figure 2). Thus, this α-helix and its immediate surround play little or no role in Chi's control of RecBCD or its promotion of recombination.

We assessed the role of the RecB tether in RecBCD nuclease activity inside cells by a second, T4 phage-based assay (see 'Materials and Methods' section for details). T4 *gene 2* mutant (*2*$^{-}$) phage formed plaques only on the *recB* null mutant; nuclease activity was maintained when the entire linker region (877–929) was deleted, demonstrating that it is not required for nuclease activity (Supplementary Figure S1 and Table 1).

In order to determine the role of the tether in Chi hotspot activity and recombination proficiency, we focused on a part of the linker, amino acids 881–899 of RecB, the 'tether' (Figure 2).

Shortening the tether by one to 19 amino acids strongly reduces Chi-dependent RecBCD activities

To determine if the tether is needed for RecBCD enzyme activities, we deleted amino acids 881–899 (Δ19) of RecB, the entire tether (Figure 2). The resulting mutant (Δ19) was recombination-deficient and had no detectable Chi activity (1.1 ± 0.1; Tables 1 and 2 and Figure 3A). These data show that the tether is essential for Chi's stimulation of recombination. The mutant retained nuclease activity, demonstrating that it is not a null mutant (Tables 1 and 2).

To determine how much of the tether is required for Chi activity, we tested RecB mutants lacking one or another part of the tether (Figures 2 and 3A; Supplementary Figure S2). For these experiments we divided the tether into two parts—the left (amino-terminus proximal) 10 amino acids and the right (carboxy-terminus proximal) 9 amino acids. We use the nomenclature ΔL or ΔR to indicate where the deletions were introduced and indicate the number of amino acids removed; the codons deleted are listed in Supplementary Figure S2 and Table S2, and the results are in Table 2 and Figure 3A. We first deleted the left 10 amino acids (ΔL10) or the right 9 amino acids (ΔR9). Each of these strongly reduced Chi hotspot activity—to 3.0 ± 0.3 for ΔL10 and to 2.1 ± 0.1 for ΔR9. More extensive deletions (Δ13–Δ18) had almost no Chi hotspot activity, ranging from 1.5 to 1.2. A shorter, one amino-acid deletion (ΔR1) significantly reduced Chi hotspot activity (to 3.3 ± 0.6; $P = 0.025$ by unpaired two-sided t-test), although ΔL1 had at most a marginal effect on Chi hotspot activity (4.6 ± 0.8). Deleting two or three amino acids, either on the left or on the right, also strongly reduced Chi hotspot activity—to 3.2 and 3.0 for ΔL2 and ΔL3, and to 2.4 and 2.3 for ΔR2 and ΔR3. Nuclease activity, however, was maintained in these RecB tether deletions, as T4 *2*$^{-}$ failed to form plaques on these mutants (Table 2). Thus, shortened tethers interfere with Chi activity but not with general nuclease activity.

Lengthening the tether by 19 or 38, but not by only 9 or 10, amino acids reduces Chi-dependent RecBCD activities

Because the tether is nearly completely stretched out in the published structures (12,34–35) (Figure 2), we wondered if

Table 1. Deleting the tether abolishes Chi activity and strongly reduces recombination proficiency

Amino acids deleted[a]	Tether length (amino acids)	Lambda cross[b]		*E. coli* Hfr cross (relative recombinant frequency)[d]	T4 2^- plaque formation[e]
		Chi hotspot activity	Relative recombinant frequency[c]		
None (wt)	19	5.1 ± 0.09	≡1	≡1	–
None (null)		0.99 ± 0.02	0.02 ± 0.001	0.003 ± 0.001	+
877–929	0	1.1 ± 0.06	0.02 ± 0.003	0.02 ± 0.005	–
913–922 (Δ α-helix)	19	4.2 ± 0.3	0.85 ± 0.1	0.8 ± 0.1	–
881–899 (Δ19)	0	1.1 ± 0.1	0.05 ± 0.01	0.02 ± 0.006	–

[a]Strains are transformants of strain V2831 (*ΔrecBCD2371*) with the indicated *recB* allele on derivatives of plasmid pSA607 (*recBC^{2773}D*) (see Supplementary Table S2). The *recBCD* null is *recB21* (IS*186* insertion) (14).
[b]Data are the mean ± SEM from three to 14 independent experiments.
[c]J^+ R^+ recombinant frequencies relative to that for the *recBCD*$^+$ (wt) strain in concurrent crosses (11.1 ± 0.07% over all experiments). Data are mean ± SEM from three to 14 independent experiments.
[d]His$^+$ [Str^R] recombinant frequencies relative to that for the *recBCD*$^+$ (wt) strain in the concurrent crosses (4.9 ± 0.29% over all experiments).
[e]+, many plaques; –, no visible plaques. See Supplementary Figure S1 for examples.

Table 2. Summary of RecB tether mutant activities

recB mutation[a]	Genetic activity			Enzymatic activity		
	T4 2^- plaque formation[b]	Chi hotspot activity	Relative Hfr recombinant frequency[c]	ATP-dep nuclease activity (units/mg protein)[d]	DNA unwinding[e]	Chi cutting[e]
recBCD$^+$	–	5.1 ± 0.09	≡1	≡1	+	+
recB21	+	0.99 ± 0.02	0.005	0.06	–	–
Δα-helix	–	4.2 ± 0.3	0.93	0.87	+	+
Δ877–929	–	1.1 ± 0.06	0.02	0.12	+	–
Δ19 (881–899)	–	1.1 ± 0.1	0.02	0.17	+	–
ΔR9 (891–899)	–	2.1 ± 0.1	0.14	0.17	+	–
ΔR1 (893)	–	3.3 ± 0.6	0.47	0.25	+	+ weak
ΔR2 (893–894)	–	2.4 ± 0.3	0.21	0.28	+	+ weak
ΔR3 (893–895)	–	2.3 ± 0.2	0.25	0.23	+	–
ΔL10 (881–890)	–	3.0 ± 0.3	0.28	0.31	+	+ weak
ΔL1 (882)	–	4.6 ± 0.8	0.52	0.74	+	+
ΔL2 (882–883)	–	3.2 ± 0.2	0.24	0.39	+	+
ΔL3 (882–884)	–	3.0 ± 0.2	0.24	0.37	+	+ weak
7 glycine	–	4.4 ± 0.29	0.64	0.58	+	+
9 glycine	–	1.7 ± 0.15	0.22	0.42	+	–
16 glycine	–	1.5 ± 0.15	0.10	0.26	+	–
6 proline	–	4.8 ± 0.4	0.78	0.54	+	+
10 proline	–	2.3 ± 0.36	0.19	0.23	+	+ weak
16 proline	–	2.6 ± 0.6	0.22	0.40	+	+ weak
Random left 1	–	4.4 ± 0.14	0.35	0.67	+	+
Random left 2	–	4.8, 3.7	0.77	0.51	+	+
Random right 1	–	4.6, 4.3	0.60	0.69	+	+
Addition (+Prot, Vib)	–	3.3 ± 0.4	0.36	0.43	+	+

[a]The indicated tether mutation was on a derivative of plasmid pSA607 (*recBC^{2773}D*) in strain V2831 (*ΔrecBCD2371*). Data are from Table 1, Supplementary Tables S4 and S6, and Supplementary Figure S2.
[b]+, many plaques; –, no visible plaques. See Supplementary Figure S1 for examples.
[c]His$^+$ [Str^R] recombinant frequencies relative to that for the *recBCD*$^+$ (wt) strain in the concurrent Hfr crosses (4.9 ± 0.29% over all experiments).
[d]Specific activity in units of ATP-dependent dsDNA nuclease/mg of extract protein (40) relative to *recBCD*$^+$ extract (470 units/mg). Data are from Table 1 or from two to four independent extracts except for Δ877–929 (one assay).
[e]Activity in extract assays (Figure 4 and Supplementary Figure S6). +, activity present. –, activity absent.

a longer tether might be functional and perhaps give Chi hotspot activity greater than that in wt. We therefore added 9 (R) or 10 (L) amino acids by non-tandemly duplicating the right or left parts of the tether (Supplementary Table S3) producing RLR (28 amino acids) and LRL (29 amino acids). These mutants were nearly indistinguishable from wt (Figure 3B and Supplementary Table S4). We next inserted the 19 amino acids of the RecB tethers in two other *Enterobacteriaceae* related to *E. coli—Proteus mirabilis* and *Vibrio harveyi,* whose RecBCD enzymes are controlled by Chi (5′ GCTGGTGG 3′) (43,44). These insertion mutants, with 38-amino-acid tethers, had significantly reduced Chi hotspot activity (3.6 and 3.1, respectively) and recombination proficiency (Figure 3B, Supplementary Table S4). Inserting both foreign tethers, to make the tether 57 amino acids long, reduced Chi activity and recombination proficiency to about the same level as that of RecBCD with the 38-amino-acid tethers (Figure 3B and Supplementary Table S4). These results demonstrate that the tether can be lengthened somewhat without alteration of Chi's control,

but a too-long tether significantly reduces Chi's control of RecBCD. None of the mutants reported here increased Chi hotspot activity.

Randomization of the tether amino acids has little effect on Chi-dependent RecBCD activities

Among RecBCD enzymes of *Enterobacteriaceae*, including *E. coli*, the amino acid sequences of the tether and its immediate surround are more variable than those of all other regions of RecB. In three species most closely related to *E. coli* the tether shares only 37% identity with that of *E. coli,* whereas the rest of RecB shares 81% identity (Supplementary Figure S3). In eleven species spanning the range of *Enterobacteriaceae* the tether shares only 5% identity, whereas the rest of RecB shares 31% identity (Supplementary Figure S4), even though these and all other *Enterobacteriaceae* tested retain Chi-dependent genetic and enzymatic activities (43,44). To test the notion that the amino acid sequence of the tether is not critical, we randomized the amino acid sequence of the left or right portions while keeping its length constant. We tested six randomizations of the left portion and two of the right and found all to be indistinguishable from wt (Figure 3C, Table 2 and Supplementary Tables S3 and 4). As a further test, we replaced the 19 amino acids of the *E. coli* tether with those of the *V. harveyi* or *P. mirabilis* RecB tether; these two *Enterobacteriaceae* are the among the most distantly related to *E. coli* of the 19 species our lab examined and found to have Chi hotspot activity or Chi cutting activity or both (43,44). The amino acid sequences of the replacement tethers were only 21% (*P. mirabilis*) or 32% (*V. harveyi*) identical to that of *E. coli,* but the Chi activity of each substitution was similar to that of wt (Figure 3C; Supplementary Tables S3-4 and Figure S4). We conclude that the exact amino acid sequence of the tether is not critical.

Substitutions to either increase or decrease the flexibility of the tether dramatically reduce Chi-dependent RecBCD activities

Although the data above suggested that the amino-acid sequence of the tether is not important, we reasoned that making it potentially either stiffer or more flexible might impede activity if the nuclease must swing on the tether (Figure 1D). Proline residues have more limited rotational capacity, and glycine residues greater, than other amino acids. The wt tether has just one proline and one glycine. Therefore, we replaced increasing numbers of amino acids in the tether with proline or glycine. In the first set of mutants, we introduced two to nine proline residues at evenly spaced intervals to avoid possible difficulties of translating repetitive proline codons (Supplementary Table S5) (45,46). In subsequent mutants, proline residues were in clusters, but this had no apparent effect on RecBCD abundance (Supplementary Figure S5). Up to six prolines had little effect on Chi hotspot activity, but 10–16 prolines strongly reduced Chi activity (to 2.2–2.9) (Figure 3D and Supplementary Table S6). Similarly, up to 7 glycines had little effect, but 9–16 glycines strongly reduced Chi activity even more (to 1.7–2.4; Figure 3E and Supplementary Table S6). These results show that the tether amino-acid composition, like the tether length, can strongly affect the ability of RecBCD to respond to Chi and to promote recombination.

RecBCD enzymatic activities coincide with the genetic activities of the tether mutants

As a more direct test of the role of the tether in Chi's control of RecBCD enzyme, we prepared cell-free extracts of selected tether mutants and assayed general (Chi-independent) nuclease, DNA unwinding and Chi-cutting activities. Because all of the mutants blocked T4 2^- plaque-formation in genetic assays, we expected the extracts to have ATP-dependent nuclease activity. We expected extracts from recombination-proficient mutants with high Chi genetic activity also to unwind DNA and cut DNA at Chi. The results of the assays were largely in accord with these expectations (Table 2, Figure 4, and Supplementary Figure S6). All of the RecB tether mutants tested had ATP-dependent ds nuclease activities that ranged from 20% to 70% of the specific activity of wt *recBCD*$^+$ extracts, consistent with their ability to block plaque-formation by phage T4 2^- (Table 2).

DNA unwinding and Chi-cutting activities were monitored on linearized pBR322 with Chi (χ^+F) or without Chi (χ^o). As shown in Figure 4A, extract from wt (*recBCD*$^+$) cells, but not from *recBCD*$^-$ cells, unwound the ds DNA substrate, producing ss DNA and cut some of the unwound DNA near Chi to produce an additional ss DNA species that was dependent on the χ^+F allele. All of the tether mutant extracts tested unwound DNA (Figure 4 and Supplementary Figure S6). With one exception (ΔR3) Chi cutting activity was observed in extracts from mutants that had Chi hotspot activities of 2.3 or greater, but it was weak from mutants with hotspot activity below 3.0 (Supplementary Figure S6). We did not observe Chi-cutting in the five tested mutants with hotspot activities of 2.1 or less (Table 2 and Supplementary Figure S6).

We examined RecBCD enzyme and its polypeptides in the mutants by western blots to determine whether the altered tethers blocked enzyme assembly or polypeptide expression or stability. As shown in Figure 4B and Supplementary Figure S5, and as expected from the similarity in unwinding activity in the extracts, on denaturing gels there was little difference in the amount of RecB or RecC polypeptides detected in wt or the tether mutant extracts whether or not they had Chi-cutting activity. On non-denaturing gels the vast majority of the enzyme was detected as heterotrimer (RecBCD). In addition, a small amount of slowly migrating RecBCD was detected in extracts of the wt and of the mutants tested, as reported previously in some preparations of purified RecBCD (47). This material may be a dimeric form of the enzyme $(RecBCD)_2$, perhaps held by the nuclease domains of two RecBCD monomers switching partners. Apparent dimers have less activity than monomers (47). Coupled with their low abundance, we suspect these forms have little if any physiological significance.

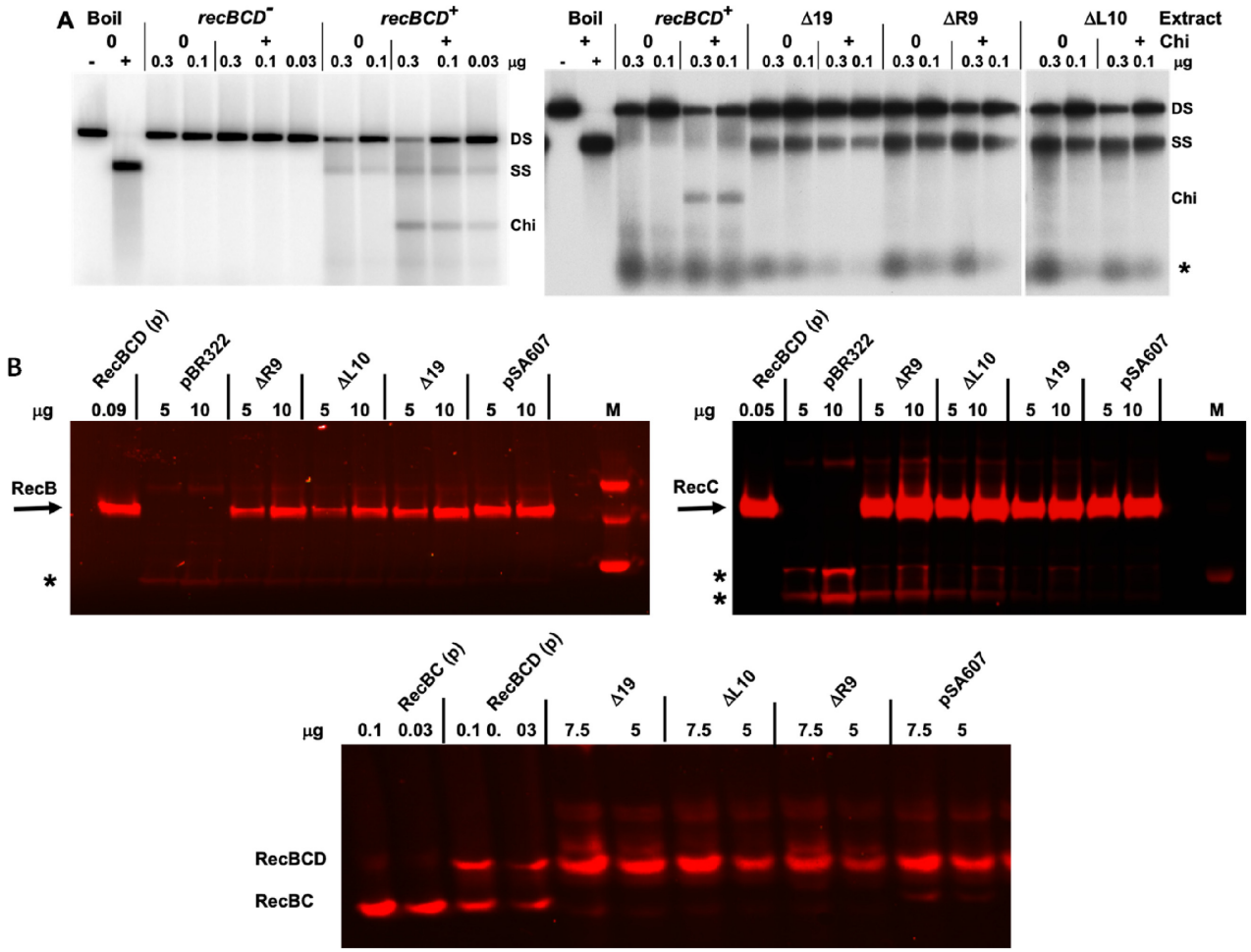

Figure 4. The RecB tether is required for full Chi-cutting activity but not enzyme assembly or DNA unwinding. Extracts were prepared from late log-phase cells of strain V2831 (*ΔrecBCD2731*) containing derivatives of plasmid pSA607 (*recBC*^{2773}*D*) with the indicated *recB* tether deletion. (**A**) DNA unwinding and Chi cutting were assayed using HindIII-linearized ^{32}P-labeled pBR322 χ^{o} or χ^{+}*F225* DNA. The indicated amount of extract protein, per 15 μl reaction, was reacted with the DNA substrate (0.8 nM) at 37° for 2 min and the products separated on a 0.9% agarose gel. The positions of dsDNA substrate (DS), unwound ssDNA (SS; boiled), the product of Chi-dependent cutting (Chi), and limit-digest oligonucleotides (*) are shown. (**B**) RecB and RecC polypeptides and native forms of RecBCD enzyme in extracts of RecB tether mutants were separated on 3–8% Tris-acetate native or SDS denaturing polyacrylamide gels and detected by Western blots using the indicated polyclonal antibodies. Purified (p) RecBCD or RecBC enzyme was run as a marker. The lane with size markers (150, 120, and 65 kDa, top to bottom) is indicated (M). The migrations of RecB and RecC polypeptides and of RecBCD heterotrimer and RecBC heterodimer are indicated. Cross-reacting polypeptides present in extracts of V2831 containing pBR322 but lacking RecBCD are indicated (*). See Supplementary Figures S5 and S6 for additional data.

Recombination frequency is reduced in direct proportion to Chi hotspot reduction

We assayed overall recombination proficiency of the RecB tether mutants in both the lambda vegetative crosses above and in *E. coli* Hfr crosses. The recombination proficiency paralleled that of Chi hotspot activity (Figure 5). In both types of crosses, mutants with high Chi activity had recombination proficiencies indistinguishable from that of wt, and those with low Chi activity had recombination proficiencies near that of the control null mutant *recB21*. Other mutants had intermediate values for these measures.

Remarkably, the data show that recombination proficiency was a linear function of the hotspot activity, although for lambda recombination the intercept was not at 0. The residual lambda recombination proficiency in the most severe RecB mutant (deletion of the entire tether) was about the same as that in the *recB21* null mutant; other recombination pathways apparently promote low-level recombination in vegetative lambda crosses. These residual pathways are not the lambda Red pathway or the *E. coli* RecF pathway, because the strains used here have null mutations in genes essential for these pathways (48). The residual recombination may result from ss DNA at replication forks; i.e., copy-choice recombination, or break-induced replication (Figure 1A, bottom right). Our results show that Chi hotspot activity is a limiting factor for homologous recombination by the wt (RecBCD) pathway.

DISCUSSION

The genetic and enzymatic results presented here support the nuclease-swing model for the control of RecBCD enzyme by Chi recombination hotspots (Figure 1D) (33). In

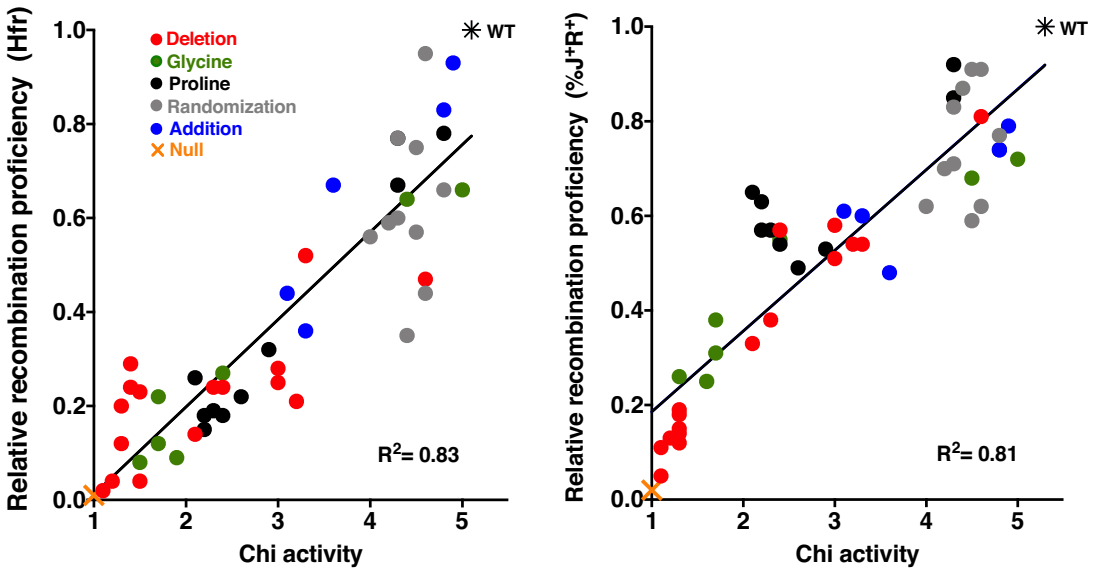

Figure 5. Recombination proficiency in the RecBCD pathway is a linear function of Chi hotspot activity. Recombination proficiency was measured in *Escherichia coli* Hfr crosses (left) and lambda hotspot crosses (right). Data are relative to wt values and are colored according to the type of tether mutation in the key at top left. Data are from Tables 1 and 2, Supplementary Tables S4 and S6, and Figure 3. Regression lines are derived from least-squares analysis.

this model, RecBCD's encounter with Chi triggers the nuclease domain to swing on its 19-amino-acid tether into a position where it can cut the DNA near Chi and begin loading RecA strand-exchange protein onto the newly created end. As predicted, changing the tether can nearly abolish Chi activity or leave it intact, depending on the nature of the tether change. Major changes, including shortening it or making it stiffer or more flexible, reduced or abolished Chi activity, indicating the essential role of the tether. Lesser changes, including changing the amino acid at each position, left Chi activity intact, consistent with the tether being the most highly variable part of RecB among the *Enterobacteriaceae* enzymes, of which all tested versions respond to Chi (43,44). Below, we discuss the role of the tether in Chi's control of RecBCD.

A central role for the RecB tether in the RecBCD–Chi interaction

Several features of the tether are important for RecBCD to respond to Chi. First, it must be the proper length. Shortening the wt 19-amino-acid tether by as little as one amino acid (ΔR1) significantly reduced Chi hotspot activity (Figure 3A). In the published crystal and cryo-EM structures (12,34–35) (Figure 2), the tether appears stretched: the length of the slightly curved tether is ∼5.5 nm, nearly the maximum distance possible if stretched straight (6.8 nm). The significant effect of reducing the tether's length by one amino acid suggests, within the context of the model in Figure 1D, that the RecB helicase domain and the RecC tunnel exit are not free to move much to accommodate this shortening. Shortening the tether even more, by as many as three amino acids, however, does leave significant, but strongly reduced, Chi hotspot activity. We infer that optimal response to Chi requires the full (wt) length tether. This view is consistent with *none* of the 49 mutant tethers studied here having *increased* Chi hotspot activity. It is also consistent with all of the 15 *Enterobacteriaceae* RecBCD enzymes reported to respond to Chi having tethers 19 amino acids long, even though their tether sequences are highly variable (Supplementary Figures S3 and S4). We suppose that evolution has selected tethers to provide maximal Chi activity and that length is more important than amino acid sequence.

The tether can be lengthened by as many as 10 amino acids without significant loss of activity (Figure 3B). We infer that the nuclease domain can assume its proper position for the response to Chi with the tether looped out, or buckled, but to a limited extent: lengthening the tether by additional 19 or more amino acids, however, significantly reduced Chi hotspot activity. Tethers too long may not provide the force, discussed below, that we suppose is required to move the nuclease domain in response to Chi.

Although limited randomization of the amino acid sequence of the tether, including substitution of tethers from other species, had no significant effect, substitution with ten or more proline or nine or more glycine residues strongly reduced Chi hotspot activity (Figure 3D and E). We suppose that multiple glycines make the tether too flexible, for glycine has more freedom of rotation about its α-carbon bonds than other amino acids. Conversely, multiple prolines make the tether too stiff, for one of its α-carbon bonds cannot rotate. From these results, we suppose that the tether transmits a force, discussed below, from the RecB helicase domain to the RecB nuclease domain to place the nuclease domain in the correct position to allow Chi cutting. The tether must be sufficiently flexible, but not too flexible, to transmit this force.

A molecular model for Chi-dependent nuclease swinging

Based on the considerations above, we propose the following scheme for the tether in Chi's control of RecBCD's activ-

ities. As shown in Figure 1D, the nuclease domain occupies either of two sites—at the exit of the RecC tunnel, where it has access to the DNA strand with a 3′ end where RecBCD began unwinding, and far away from this exit, on the other ('left') side of the RecC subunit, where it cannot cut the 3′-ended strand. We suppose that the interface between the RecB nuclease domain and each of the two RecC sites provides an intrinsic attraction, much like a magnet binding to either of two sites. To move the nuclease from one site to the other requires a force great enough to overcome that attraction. We propose that this abrupt pulling force is provided by the RecB helicase domain, an ATPase, and is transmitted to the RecB nuclease domain via the tether. A tether with too many glycines may be too flexible to transmit this force, whereas a tether with too many prolines may be too stiff to allow the nuclease domain to readily move from the left side to the RecC tunnel exit. No particular amino acid sequence is required, but the tether must be long enough to allow the nuclease domain to contact the tunnel exit. Shortening the tether by even one amino acid could significantly reduce the ability of the nuclease to approach the exit. Lengthening the tether would have no effect until the tether was so long that the helicase domain could not readily pull on the nuclease domain to dislodge it from the left side of RecC.

According to this model, the nuclease domain would have a residence time at each site. To account for the conformational changes reported by Taylor et al. (33), we propose that the residence time at the RecC tunnel exit, versus its residence time on the left, is influenced both by the stage of the RecBCD reaction cycle on DNA and by the reaction conditions. In the absence of DNA, the nuclease resides primarily at the tunnel exit, as in the published structures (12,34,35) (Figure 1D, part a). When DNA is added and the 3′ end enters the RecB helicase domain, that domain pulls on the tether and moves the nuclease domain to the left (Figure 1D, part b), where its residence time is greater than that at the tunnel exit. When ATP is added and unwinding begins, the nuclease domain remains primarily on the left if the ATP:Mg^{2+} ratio is high. Under this condition there is little DNA nicking during unwinding (6,19). But if the ATP:Mg^{2+} ratio is low, the nuclease domain residence time at the tunnel exit is increased, and the enzyme occasionally nicks the DNA, to produce a long oligonucleotide, as observed (19,49), and returns to the left. When Chi is encountered in the RecC tunnel under either condition, RecC signals RecD to signal the RecB subunit (Figure 1C) to pull on the tether to move the nuclease domain to the tunnel exit (Figure 1D, part c), where it nicks the DNA at Chi and loads RecA onto the ss DNA with the new 3′ end. This 3′ ended ss DNA-RecA filament then undergoes strand exchange with homologous DNA and produces Chi-stimulated recombinants.

After RecBCD has loaded RecA onto the Chi-dependent 3′ end, we imagine two possibilities. The nuclease domain may remain near the tunnel exit and continuously load more RecA onto the initial ssDNA-RecA filament. Alternatively, the nuclease domain may return to its former position on the left side of RecC and occasionally return to the tunnel exit to load more RecA. Since RecA can self-polymerize in the 5′ → 3′ direction (50–52), occasional returns would be sufficient to make the filament of multiple, contiguous RecA proteins required for efficient strand exchange (53,54). The residence time of the nuclease domain at the two sites might depend on the ATP:Mg^{2+} ratio, as before Chi: with a high ATP:Mg^{2+} ratio the residence time might be longer at the left than at the tunnel exit and result in little DNA nicking, whereas with a low ATP:Mg^{2+} ratio the residence time might be longer at the tunnel exit and result in occasional nicking of the 5′-ended strand, as observed (19). Since the initial products of RecBCD nuclease are long oligonucleotides (>0.5 to 1 kb) (18,19,49,55), the residence time at the left might be greater than that at the tunnel exit under both reaction conditions. In bacterial cells, DNA abundance is reduced in the ∼0.2–1 Mb region near an introduced DSB (65,66); this loss likely reflects unwinding by the initial RecBCD enzyme followed by action of additional RecBCD molecules or other enzymes (1).

How long does it take for the nuclease domain to swing from one position to the other? Major conformational changes of the type proposed here can take anywhere from a μs to an hour (56). We propose that it takes on the order of 1 ms for the nuclease domain to swing from the left side to the tunnel exit after Chi is encountered. RecBCD unwinds DNA at a rate of 0.3 to 2 kb per s, or 1 bp per 0.5 to 3 ms (2,9,57). We proposed above that when Chi is in the RecC tunnel, RecB is signaled to swing the nuclease from the left side of RecC to the tunnel exit and to cut the DNA. Cutting occurs to the 3′ side of, or within, the Chi octamer (6,19). At each of the two Chi sites analyzed, cutting is not at a single position; rather, it is at two or three internucleotide positions (4–6 nt to the 3′ side of Chi) with high ATP:Mg^{2+} ratio and at seven internucleotide positions (from within the Chi core sequence to 4 nt to the 3′ side) with low ATP:Mg^{2+} ratio (19). Thus, we propose that the nuclease domain swings into its cutting position in about 1 ms after Chi is encountered. Given a normal (Boltzmann) distribution of energies among the RecBCD enzymes, cutting would be spread over DNA distances traversed on the order of the time it takes for the swing. With low ATP:Mg^{2+} ratio RecBCD unwinds DNA about three to five times more rapidly than with high ATP:Mg^{2+} ratio (2,9,58–59). If swinging takes the same time under these two conditions, then with low ATP:Mg^{2+} ratio more DNA would have passed through the tunnel and the nuclease would land and cut at a broader range of internucleotide positions, consistent with cuts being distributed over a longer distance with low ATP:Mg^{2+} ratio (19). In addition, this view accounts for the cuts being farther to the 5′ side with low ATP:Mg^{2+} ratio (19).

The proposal that the swing time is about 1 ms for wt RecBCD can account for the failure to observe Chi-cut products by tether mutants that nevertheless have significant, but reduced Chi hotspot activity (Supplementary Figure S6 and Table 2). These tether mutants may take longer to swing the nuclease domain from the left to the tunnel exit. In this case, DNA unwinding would have positioned DNA far to the 5′ side of Chi at the tunnel exit by the time the nuclease domain arrived there. In accord with the proposal above, a Boltzmann distribution of energies among these RecBCD enzymes would position the nuclease at many internucleotide positions and result in a broad series of cuts and a light smear on the gel used for analysis of Chi-cut

products; these might have been below the level of detection. But the genetic intervals in which Chi can stimulate recombination and still be detected in the hotspot assay are 7.3 kb long (χ^+D *to cI*) and 9–12 kb long (χ^+76 to *J*). Cutting could thus be displaced from Chi by nearly this distance and still stimulate recombination in the interval assayed and score as readily detected hotspot activity.

Predictions of the nuclease-swing model

The model discussed above makes predictions to be tested in future work. Most notable is directly detecting the hypothesized swinging of the nuclease domain from the left side of RecC to the RecC tunnel exit. In published crystal and cryoEM structures this distance is about 6 nm (12,34–35), a distance change readily detected by Förster resonance energy transfer (60). This method might allow estimation of the residence times at the two sites, predicted above to be sensitive to the ATP:Mg^{2+} ratio and the stage of the RecBCD reaction cycle (with and without DNA; before and after encounter with Chi). It might allow assay of the time required for the nuclease domain to swing. The mutant enzymes described here provide material to directly test the effect of the tether length and composition on these residence and swinging times.

The model makes several predictions about the phenotypes of other mutant RecBCD enzymes. In accord with the discussion above, it predicts that slowing the RecB helicase, by appropriate mutation, would make all the cuts at Chi appear at one internucleotide position, likely 6 nt 3′ of the Chi octamer, the position at which cuts are observed farthest to the 3′ side of the Chi octamer (6,61). Conversely, mutations that increase the rate of the RecB helicase would result in Chi-dependent cuts being displaced 5′ of their position with wt RecBCD and spread over more internucleotide positions. Mutations in the RecC patch that increase the affinity of the patch for the nuclease domain would result in less frequent DNA cutting, perhaps both before and after Chi, especially with low ATP:Mg^{2+} ratio. Conversely, mutations that decrease the affinity would result in more frequent cutting and perhaps higher Chi hotspot activity; mutations that slow the RecB helicase might have a similar effect. [Note that wt RecBCD cuts at Chi only about 30% of the time it traverses Chi in the proper orientation (32)]. Such mutants would be valuable for both biochemical and genetic studies of the RecBCD–Chi interaction.

Comparison with other proteins containing large, movable domains

The ability of large domains to move large distances has been reported for other enzymes acting as molecular machines. Prime examples are kinesin and dynein, which move cargos along cellular filaments (62). These ATP-dependent motor proteins take steps ranging from ∼8 to ∼36 nm and can move as frequently as one step per 12 ms. The mass of the moving domains are ∼34–130 kDa. These features are similar to those proposed above for the 32 kDa nuclease domain of RecBCD. The F1Fo ATP synthase is similar to a rotor inside a stator; the rotor is ∼1 nm in diameter and can spin up to ∼1 revolution per ms (63). Although the energy source is a proton gradient, rather that ATP (which it synthesizes), the speed and mass movement are comparable to those of RecBCD. These examples suggest that the RecBCD nuclease could undergo the large, rapid conformational change we propose in Figure 1D. Further tests of the type in the preceding section may support this model.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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